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TECHNICAL NOTE

# Kinetics of glomerular visceral epithelial cell phagocytosis

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The functions of the glomerular visceral epithelial cell include *a)* participation in the formation of the glomerular filtration barrier [1], *b)* the synthesis and maintenance of the glomerular basement membrane (GBM) [2], and *c)* the clearance of macromolecules from the glomerular filter. The latter characteristic has been repeatedly observed under a variety of experimental conditions. Morphologic evidence of the phagocytosis of filtered macromolecular dextran by epithelial cells has been demonstrated in normal glomeruli [3]. The epithelial cell uptake of significant amounts of larger tracers, such as ferritin, however, has been achieved only after rendering rats nephrotic, such as with administration of puromycin aminonucleoside [4]. Studies carried out in various experimental proteinuric states have also shown that some of the abnormally filtered albumin is taken up by the epithelial cells and sequestered in phagosomes within these cells [5]. Since macromolecular tracers are largely excluded from the lamina rara externa, the quantitation of epithelial cell phagocytic activity has hitherto not been accomplished.

Perfusion of rat kidney with protamine sulfate followed by a neutralizing dose of heparin results in the formation of electron-dense protamine-heparin aggregates along the GBM [6]. After the in vivo aggregation and localization of protamine-heparin complexes in the lamina rara externa, the GBM maintains apparently normal structural and functional characteristics as assessed by electron microscopy and urinary protein excretion [7]. Morphologic observations suggest that the aggregates are cleared by epithelial cell phagocytosis [7]. Using the protamine-heparin aggregates as tracers and a morphometric technique, we have studied the kinetics of protamine-heparin aggregate disappearance in the normal rat kidney.

Sprague Dawley rats weighing 200 to 250 g were injected via the tail vein with protamine (Eli Lilly and Company, Indianapolis, Indiana), 3 mg/100 g of body wt, followed 30 to 60 sec later by heparin (beef lung sodium heparin, Upjohn Company, Kalamazoo, Michigan), 300 U/100 g of body wt. Sequential kidney biopsies were performed through a flank incision under ether anesthesia at 15 min, 1, 2, 5, 8, 10, and 12 hr after the injection. Tissue blocks were fixed by immersion in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH, 7.4) and processed as previously described [7]. Three glomeruli from each biopsy were thin-sectioned and 20 to 30 fields were photographed in a Philips electron microscope 300 and printed at a final magnification of $\times 6800$. Micrographs were then mounted on a Graph/Pen GP-3 tablet (Science Accessory Corporation) equipped with a sonic digitizer and sensors along the $X$ and $Y$ axis. The basal laminae in each electron micrograph were traced with the sonic digitizer, which was constantly monitored by the sensors. The sensors were interfaced with a programmed minicomputer (Hewlett-Packard 9830A with a 4K memory) which translated the tracing on the micrographs into the length of the capillary perimeter. The aggregates in the lamina rara externa were counted, and their number was fed into the program which determined the numerical density as the number of aggregates per 100 $\mu$ of GBM.

Protamine-heparin aggregates, being 300 to 600 Å in diameter [7], appear to be too large to simply pass through the epithelial slits and into the urinary space. The possibility that simple passage of aggregates through the slits, along with the glomerular filtrate, could account for their disappearance has nevertheless remained a consideration. We, therefore, examined the disappearance of the aggregates from the GBM after acute ureteric obstruction. As

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an acute fall in the glomerular filtration rate occurs within minutes of obstruction [8], this experiment allows the determination of whether aggregate disappearance is dependent upon normal filtrate flow. One group of animals underwent unilateral ureteric ligation 5 min after the injection of protamine and heparin. The obstructed and the contralateral kidneys were sequentially biopsied as mentioned above, the unobstructed kidneys serving as controls.

In all the experimental groups, the protamine-heparin aggregates appear to be taken up by the epithelial cells as soon as they aggregate and localize in the lamina rara externa of the GBM [7]. Fifteen minutes after i.v. injection of protamine followed by heparin, aggregates are identified in the lamina rara externa, surrounded by invaginations of the epithelial cell plasma membrane (Fig. 1a) and within intracellular vesicles in the foot processes (Fig. 1b). Twelve hours after injection, aggregate clearance from the lamina rara externa of the GBM was complete in normal kidneys as well as in the obstructed kidneys and their contralateral controls.

The numerical densities of protamine-heparin aggregates 15 min after injection are given in Table 1. The initial deposition of aggregates in the lamina rara externa, as seen from these numbers, is uniform in each group, but it varies. It is dependent on a variety of factors such as renal blood flow and surgical manipulation. To construct disappearance curves, the means of the numerical densities at each time-interval were expressed as the percentage of the numerical density at 15 min and were plotted on a semilogarithmic scale against time. The disappearance of aggregates in each group is described by a curve which is nearly straight (Fig. 2). To test for linearity, the regression coefficients of the first four points and the last four points in each group were compared by paired analysis. The deviation in all three groups was not statistically significant. Further supportive evidence for linearity is provided by the $r^2$ values which are $>0.97$ in all three groups. Paired statistical analysis showed no significant difference between means of the percentages of the numerical densities among the three groups. The

**Table 1.** Initial deposition of protamine-heparin aggregates in the lamina rara externa

| Kidneys | Numerical density at 15 min[a] |
|---|---|
| Normal | 138.56 ± (SD) 11.36 |
| Obstructed | 89.38 ± (SD) 11.36 |
| Controls | 91.58 ± (SD) 9.14 |

[a] Number of agrregates/100 $\mu$ of basal lamina.

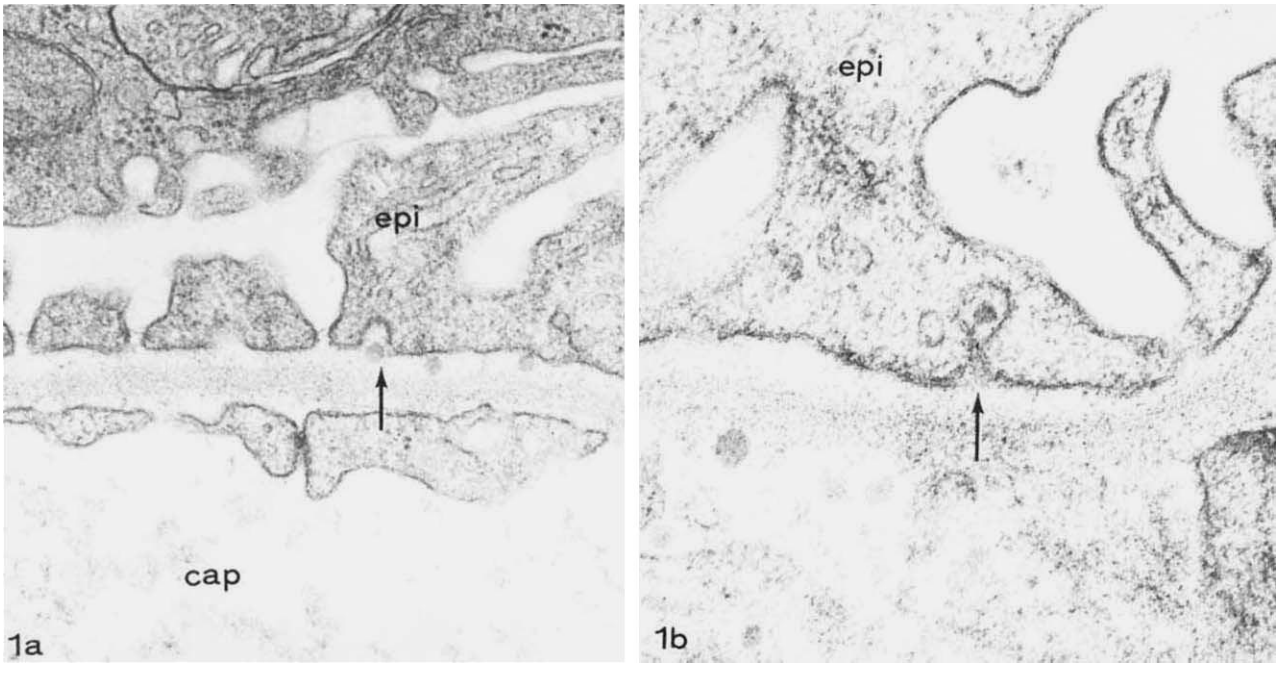


**Fig. 1.** *Protamine-heparin aggregate deposition and phagocytosis. a)* Fifteen minutes after the injection of protamine and heparin, spherical electron dense aggregates are present in the glomerular capillary wall (magnification ×50,000). An aggregate in the lamina rara externa (*arrow*) is present with an invagination of the plasma membrane of an epithelial foot process. *b)* After the same time-interval, an electron-dense aggregate is seen in the process of phagocytosis by the epithelial cell (*arrow*) with the opposing membranes of the endocytic vacuole nearly fused (magnification ×57,000). *Epi* denotes epithelial cell; *cap* denotes capillary lumen.

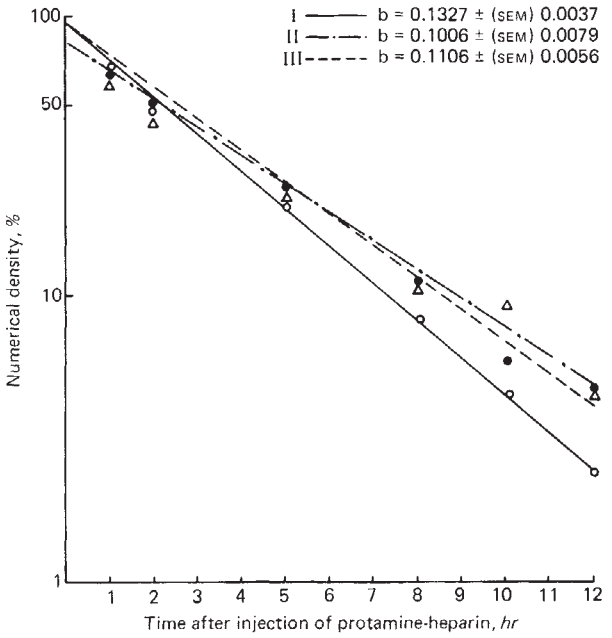


**Fig. 2.** *Protamine-heparin aggregate disappearance curves. Open circles* (○) denote means in normal animals (I), *triangles* (△) in obstructed animals (II), and *closed circles* (●) in contralateral controls (III); *b* denotes the regression coefficients ± SEM. $N = 3$ animals in each group.

half disappearance time of aggregates from the lamina rara externa was 1.98 hr in the normal kidneys, 1.95 hr in the obstructed kidneys, and 2.27 hr in the contralateral controls. There was no significant difference among the three groups when tested by Student's *t* test.

These results indicate that protamine-heparin aggregates which deposit in the lamina rara externa of the normal rat GBM, under these experimental conditions, are cleared according to first order kinetics. The constant rate of disappearance of aggregates throughout the experiment is compatible with a single biological mechanism being responsible for their clearance. Since a reduction of the glomerular filtration rate achieved by acute ureteric ligation did not affect the rate of aggregate disappearance, it seems unlikely that their clearance is a passive filtration-flow-related phenomenon. In vivo measurements of kinetics of phagocytosis of the mononuclear phagocytic system (reticuloendothelial system) [9] and in vitro measurements of initial rates of phagocytosis in polymorphonuclear leukocytes [10] have shown kinetics of uptake similar to the kinetics of aggregate disappearance in the present experiments. Thus, the disappearance curves in the present experiments further support the morphological observation that aggregate clearance is accomplished by epithelial cell phagocytosis.

Although the perfusion of kidneys with protamine sulfate was shown to alter glomerular capillary structure [6] and to transiently increase its permeability to albumin [11], the immediate neutralization of protamine sulfate with heparin reverses the structural changes to normal [6]. In addition, glomerular permeability, as assessed by urinary protein excretion, remains intact after repeated administration of protamine followed by heparin [7]. The introduction of the protamine-heparin aggregates into the lamina rara externa of the GBM via the systemic circulation seems therefore to result in no permanent alteration in function or morphology. The aggregates' sizes appear to prevent their passage into the urinary space via the epithelial filtration slits. We interpret the disappearance curve described as allowing a quantitative analysis of phagocytic activity in vivo. We propose that this technique can serve as a model for the study of the glomerular epithelial cell phagocytic function in the normal and in disease states.

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